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# Rhelogical, dermal wound healing and *in vitro* antioxidant properties of exopolysaccharide hydrogel from *Pseudomonas stutzeri* AS22

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ABSTRACT

The *in vitro* antioxidant activity and the *in vivo* wound healing performance of the exopolysaccharide EPS22, produced by *Pseudomonas stutzeri* AS22, were investigated.

Antioxidant activity was evaluated by three different tests. The scavenging effect on DPPH radicals at a concentration of 1 mg/ml was 80 ± 1.41%. The reducing power reached a maximum of 1.26 ± 0.02 at 2 mg/ml. Moreover, EPS22 showed good chelating ability and chelated almost 88.5 ± 0.7% of ferrous ions at 0.75 mg/ml.

The rheological characterization of EPS22 gel (0.5%) showed a pseudoplastic behavior, high elasticity, good mechanical strength and stability with high water-absorption ability.

The application of the EPS22 gel on dermal full-thickness excision wounds in a rat model every two days, enhanced significantly wound healing activity and a total closure was achieved after 12 days of wound induction. Further, histological examination of biopsies showed advanced tissue regeneration, characterized by the presence of well-organized stratum of both derma and epidermis.



## 1. Introduction

Bacterial exopolysaccharides (EPSs) have emerged as an important class of biopolymers which are gradually becoming economically competitive with their animal (chitin and chitosan) [1], plant (cellulose, pectin and starch) and algal-derived counterparts (agar, alginate and carrageenan) [2]. In addition to their season independency, biodegradability, biocompatibility, bioadhesivity, chemical and structural diversity, the possibility of using genetically modified microorganisms under controlled fermentation conditions may result in the production of new exopolysaccharides with novel improved properties which will open up new areas of industrial applications and thus increase their demand [3]. Some of these applications include their use in the pharmaceutical, medical and cosmetic fields, due to their rheological and physical features and particularly their specific biological properties like antioxidant [4] and wound healing [5] activities.

In fact, wound healing, a significant concern in many pathologies (post-surgeries, burns, scars), is a dynamic complex process involving biochemical and physiological phenomena such as inflammation, proliferation, and remodeling, behaving in a harmonious way to ensure tissue repair [6]. However, this process may be hampered by the production of high level of free radicals during the inflammatory phase, that if not controlled by the host's antioxidative capacity, leads to the inhibition of cell migration and proliferation, and thus the damage of the wound surrounding tissue [7].

With the aim of overcoming the previously described drawbacks, advanced research projects were carried out to find novel bioactive compounds with strong antioxidant activity that will promote proper tissue repair and improve impaired wound healing.

The objective of this study was to evaluate the antioxidant properties of an exopolysaccharide EPS22, produced by *Pseudomonas stutzeri* AS22. The EPS22 performance as a hydrogel wound dressing was also evaluated through rheological characterization,

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moisture-absorption capacity test and *in vivo* wound healing using excision wound model.

## 2. Materials and methods

### 2.1. Reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH), ethylene diamine tetra-acetic acid (EDTA), ferrozine and trichloroacetic acid (TCA), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Butylated-hydroxyanisole (BHA) and all other chemicals were of analytical grade.

For wound healing evaluation, the "CICAFLORA" was used as the reference product. It consists in an oil-water emulsion that contains Mimosa as the main active component and is marketed in a cream form.

### 2.2. Production and purification of the EPS22

The production of exopolysaccharide EPS22 by *P. stutzeri* AS22 was performed in liquid medium composed of (g/l): starch 10, yeast extract 5, $MgSO_4$ (7 $H_2O$) 0.1, $K_2HPO_4$ 1.4, $KH_2PO_4$ 0.7, and NaCl 0.5, in 250 ml conical flasks with 25 ml of liquid medium. The fermentation temperature, initial pH value were 30 °C and 8.0, respectively. The culture was conducted in a rotator shaker at 200 rpm for 24 h.

Under these conditions, *P. stutzeri* was able to produce approximately 1.45 g/l of EPS22, estimated calorimetrically. The exopolysaccharide EPS22 was recovered from the culture broth by centrifugation and filtration. It was successively purified by ultra filtration (using a 300 kDa cut-off membrane), dialysis against distilled water, and lyophilized [8].

### 2.3. Scanning electron microscopic (SEM) analysis

The scanning electron microscopy was performed using a JEOL JSM-6100 (Tokyo, Japan) instrument. The lyophilized EPS22 sample (5 mg) was fixed to the double-sided adhesive coated aluminum SEM stubs. After being frozen under liquid nitrogen, fractured, mounted and coated with a gold/palladium layer (6 nm thick) on a JEOL JFC-1100E ion sputter coater, specimens were finally observed. SEM micrographs were obtained using an accelerated voltage of 10.0 kV in secondary electron mode.

### 2.4. Antioxidant activities of EPS22

#### 2.4.1. DPPH radical-scavenging assay

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging potential of the polysaccharide EPS22 at different concentrations (1–5 mg/ml) was determined according to the reports of Bersuder et al. [9].

Radical-scavenging activity was expressed as the inhibition percentage and was calculated using the equation of DPPH radical-scavenging activity.

$$\text{Inhibition of DPPH radical}\,(\%) = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

where Abs control is the absorbance of the control reaction and Abs sample is the absorbance of EPS22/standard BHA samples. The $IC_{50}$ value (mg sample/ml) is the effective concentration at which the DPPH radicals were scavenged by 50%. The test was carried out in duplicate.

#### 2.4.2. Reducing power assay

The ability of EPS22 (1–5 mg/ml) to reduce iron (III) was determined according to the method of Yildirim et al. [10]. The $IC_{50}$ value (mg sample/ml) is the effective concentration at which the absorbance was 0.5 for the reducing power. BHA was used for comparison and all data values are the mean of duplicate analysis.

#### 2.4.3. Metal chelating activity

The $Fe^{2+}$-chelating ability of EPS22 samples at different concentrations (1–5 mg/ml) was determined according to the method of Carter [11]. The chelating antioxidant activity was calculated according to the following formula:

$$\text{Chelating activity}\,(\%) = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

where Abs control is the absorbance of the control reaction and Abs sample is the absorbance of EPS22. Ethylene diamine tetra acetic acid (EDTA) was used as a positive control and the test was carried out in duplicate.

### 2.5. Preparation and characterization of EPS22 hydrogel

#### 2.5.1. Preparation of the EPS22 hydrogel material

During our previous work on the structural characterization of the EPS22 exopolysaccharide from *P. stutzeri* AS22 [8], the purified EPS22 showed high gel-forming ability, occurring at a minimal gelling concentration of 0.5%. The hydrogel was prepared by dissolving the lyophilized EPS22 in a sterile saline solution, to give a final concentration of 5 mg/ml. The mixture was kept under agitation until a transparent hydrogel was formed.

#### 2.5.2. Rheological characterization of EPS22 hydrogel

Rheological properties of EPS22 hydrogel (0.5% in saline solution) were determined using a stress-controlled rheometer AR2000 from TA Instruments. 1° cone steel Aluminum plates with 60 mm diameter were used and the 29 μm gap was filled with the gel. The effect of shear rate (0.01–60 $s^{-1}$) on viscosity was examined at a controlled temperature of 25 ± 1 °C, using a contraves Low Shear LS30 viscometer. The dynamic rheological measurements of EPS22 gel were also performed and the mechanical spectra were characterized by the storage modulus $G'$ and the loss modulus $G''$ as functions of frequency and temperature in the ranges of 0.01–10 Hz (at 25 °C) and 5–70 °C (1 Hz), respectively. For heating and cooling cycles, temperature was controlled with a thermostatic bath and a temperature variation rate of 5 °C/min. Continuous oscillation frequency was applied using a logarithmic sweep function with a frequency range of 0.01–10 Hz and a percent strain fixed to 12%.

In order to get more information about the response of the EPS22 hydrogel to an applied stress (shear force or temperature), a two-step stress sweep test was performed, in which the sample was subjected to an increasing (step 1) or decreasing (step 2) stress.

All data were treated by the "Advantage Rheology Data Analysis" software.

#### 2.5.3. Moisture-absorption capacity of EPS22 hydrogel

The moisture-absorption capacity of a wound dressing is one of the most important criteria for maintaining a moist environment over wound bed during the healing process.

To assess the moisture-absorption capacity of EPS22, one milliliter of the hydrogel (0.5%) was prepared in screw-capped test tubes of 10 ml capacity. After introduction of 5 ml 0.9% NaCl solution, the tubes were incubated at 37 °C. At regular time intervals, the weight of the gel was noted after removing the aqueous solution and gently blotting the gel with a filter paper until no water remained. Weights of hydrogel were noted until equilibrium swelling was reached.

The water absorption capacity (%) of the EPS22 hydrogel was derived from the mass change before and after water uptake, according to Balakrishnan et al. [12].

$$\text{Water absorption}\,(\%) = \frac{W_f - W_i}{W_i}$$

where $W_f$ and $W_i$ represent, respectively, the final and initial weights of hydrogel samples. All experiments were done in duplicate.

### 2.6. Wound healing activity test of EPS22 hydrogel

Keeping in view the antioxidant and rheological properties of EPS22, an attempt has been made to explore its potential as a hydrogel in enhancing the wound healing process.

#### 2.6.1. Experimental animals

Eighteen Wistar female rats weighing 181 ± 3.61 g were used for the experiment. They were individually housed in clean polyethylene cages under controlled conditions of 22–25 °C, 60–70% relative humidity and 12 h light–dark cycle with free access to food and water. Procedures and animal comfort were controlled by the International Guidelines for Animal Care.

#### 2.6.2. Circular excision wound model

After total anesthesia with ketamine (100 mg/kg body weight) by intramuscular injection, a full thickness circular area of approximately 200 $mm^2$ wound was created on the shaved rat's dorsal interscapular region. The wounding day was considered as day 0 and wounds, covered with a compression dressing, were treated till they were completely healed.

#### 2.6.3. Excision wounds treatment

The treatment lasted 12 days. The rats were divided into three groups consisting of six rats each. Group I was untreated and served as the control (just cleaning the wounds with a physiologic serum). Group II was treated with 0.5% (w/v) EPS22 gel and served as the test group, while group III was treated with "CICAFLORA" and served as a reference standard (positive control).

After rinsing wounds with physiologic serum, the test sample (EPS22 gel) and the reference drug (CICAFLORA) were applied, in a fine layer covering the surface of the wound, every two days till the wound was completely healed. All the rats were anaesthetized with ether, sacrificed on the 12th post-wounding day and the granulation tissue was excised from the sacrificed animals. A part of wet tissue was preserved for hydroxyproline estimation and another one was fixed in formalin 10% (v/v), embedded in paraffin and processed for histological observation.

#### 2.6.4. Wound healing evaluation parameters

To evaluate the healing process of the 3 studied groups, we relied on two clinical macroscopic criteria including qualitative (color of the wound) and quantitative criteria (wound closure rates and hydroxyproline estimation) and one microscopic criterion (histological evaluation).

*2.6.4.1. Chromatic study.* Because superficial wounds tend to lighten from red to pale pink and become more homogeneous and more consistent in texture as they heal, the chromatic evaluation of the healing process was done through daily photography of wounds. This study consists of attributing a chromatic code to the wound of each rat, (bright red = blood covering the wound), (dark red = coagulation of blood in the epidermis), (red = granulation tissue) and (pink = epithelialization phase) [13].

*2.6.4.2. Rate of wound closure and epithelialization time.* The rate of wound closure of individual animal ($n = 6$) from control and treated groups was used as an indicator of wound healing. Progressive decrease in the wound size was monitored periodically every 2 days interval using transparent graph sheet and a marker. The wound area was traced, measured and the actual value was converted into percent value taking the size of the wound at the time of wounding as 100%. Wound closure, which indicates the formation of new epithelial tissue to cover the wound, was expressed as reduction in percentage of the original wound size using the following expression:

$$\text{Wound closure}\,(\%) = \frac{A_0 - A_n}{A_0} \times 100$$

where $A_0$ and $A_n$ are the initial wound area (day 0) and wound area on day $n$, respectively.

Falling of scab (dead-tissue remnants) without any residual raw wound was taken as end point of complete epithelialization and the days required for this were taken as period of epithelialization [14].

*2.6.4.3. Hydroxyproline estimation.* Hydroxyproline content of tissue samples was estimated according to Lee and Tong [15] and values were reported as mg/g dry weight of tissue.

*2.6.4.4. Histological examination.* Following fixation, paraffin sections of 3 μm were made perpendicularly to the skin surface, including the whole thickness of the skin. Serial sections ($n = 4$) were stained with hematoxylin–eosin (HE) [16] to show the common morphology of the tissues: epithelial proliferation, granuloma tissue formation and organization, newly formed capillaries, collagenisation and scar formation in the dermis. Other sections were stained with alcian blue according to the technique of Scott and Dorling [17], to point out acid glycosaminoglycans and proteoglycans of the extracellular matrix. Sections were qualitatively assessed under light microscope and the results of the EPS22 treated group were compared with the control (none) and CICAFLORA-treated groups.

### 2.7. Statistical analysis

Data from all tests are presented as the mean ± standard deviation (SD). Student's $t$-test and one-way analysis of variance (ANOVA) were applied to ascertain significant differences between EPS22 gel-treated group and both positive and negative control groups. Differences were considered to be statistically significant at $P < 0.05$.

## 3. Results and discussion

### 3.1. Scanning electron microscopic (SEM) analysis

The scanning electron microscopy (SEM) is a fundamental tool to assess the surface morphology and complex 3D microstructure of polysaccharides. The scanning electron micrographs of the *P. stutzeri* EPS22 (Fig. 1) revealed a sponge-like structure (Fig. 1a) containing numerous small pore size (Fig. 1b) with their surfaces having a sheet appearance (Fig. 1c). Such structure makes the EPS22 suitable for a variety of applications, ranging from food industries to the field of tissue engineering and medical biological materials. This porous structure with good pore size distribution allow the EPS22, when solubilized in water-based solutions, to absorb a large amount of water, and thus making it a fast swelling system, advantageous in several applications as texturing, gelling, stabilizing and emulsifying agent. On the other hand, such 3D porous structure indicates the potential of the EPS22 to be used for drug delivery

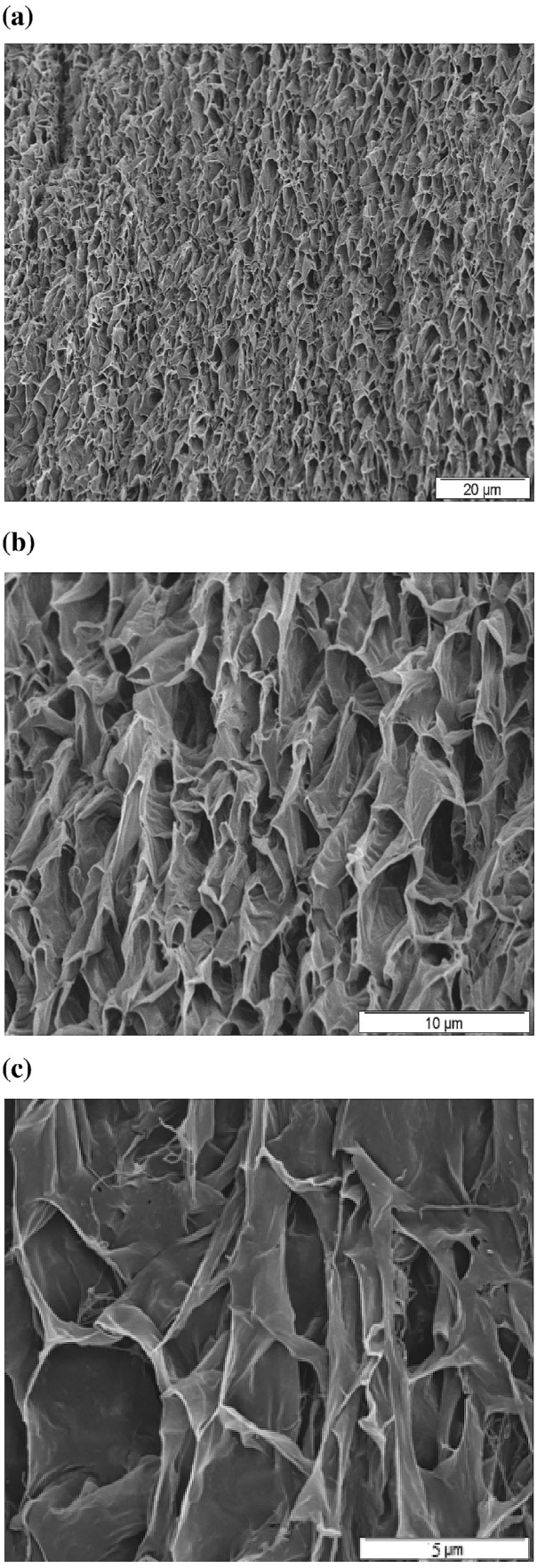


**Fig. 1.** SEM images of EPS22 exopolyscacharide from *P. stutzeri* AS22: (a) 1000×, (b) 4000×, (c) 5000×.

and/or to act as a scaffold to support cells, allow transport of nutrients and metabolic wastes and thus promote tissue development [18,19].

### 3.2. Antioxidant activities of EPS22

#### 3.2.1. DPPH radical-scavenging assay

The DPPH assay has been frequently used for evaluation of antioxidative potentials of various compounds. The DPPH radical-scavenging effects of EPS22 exopolysaccharide and BHA (used as positive control) at varying concentrations are depicted in Table 1. Although EPS22 showed a lower radical-scavenging activity than BHA at all tested concentrations, it exhibited good scavenging effect with the strongest value (80%) at a concentration of 1 mg/ml. The $IC_{50}$ value was about 450 μg/ml, slightly higher than that of BHA ($IC_{50}$ = 300 μg/ml), suggesting therefore good DPPH radicals-scavenging ability of EPS22.

According to previous studies, the effect of polysaccharides on DPPH radical scavenging was believed to be due to their hydrogen donating ability. Lo et al. [20] revealed a significant relationship between monosaccharide composition and scavenging ability and showed that the levels of arabinose, mannose and glucose in the polysaccharides are important facets of the scavenging ability. Chen et al. [21] reported that the hydroxyl groups from the monosaccharide compositions of polysaccharides and their side chain glycosidic linkages can act as electron donors to bind radicals and radical ions, and, consequently, terminate radical chain reactions.

According to these observations and our experimental results, it could be inferred that the high DPPH radicals scavenging activity of EPS22 might be related to its monosaccharide composition where glucose and mannose are the major components of the EPS22 backbone structure [8] that could react with free radicals to convert them to stable diamagnetic molecules.

#### 3.2.2. Reducing power assay

The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing powers of EPS22, as well as BHA, as a function of their concentrations are shown in Table 1. The reducing power of EPS22 increased with increasing concentrations and reached a maximum of 1.26 ± 0.02 at a dose of 2 mg/ml. Although the synthetic antioxidant BHA has better reductive capacity than EPS22, this biopolymer revealed good reductive potency with low $IC_{50}$ value of 700 μg/ml.

It has been reported that exopolysaccharides produced by *Pseudomonas fluorescens* WR-1 [22] and *Pseudomonas* PF-6 [4] showed moderate reducing powers of approximately 0.5 at 1 mg/ml and 0.7 at 2 mg/ml, respectively. Moreover, the $IC_{50}$ values were 1 mg/ml for the EPS of *P. fluorescens* WR-1 and 1.5 mg/ml for the EPS of *Pseudomonas* PF-6, which are higher than that of EPS22 (0.7 mg/ml), thus suggesting interesting reductive capacity of *P. stutzeri* EPS22.

In addition, the reductive ability of a polysaccharide may be influenced by its chemical composition especially the reductive nature of its constitutive monosaccharides [23]. Therefore, this finding may reflect the potent reducing power of EPS22, which was found to consist mainly of glucose and mannose [8], well known reductive agents that have a hidden aldehyde moiety.

#### 3.2.3. Metal chelating activity

Ferrous ion ($Fe^{2+}$) is the most powerful pro-oxidant among metal ions. The $Fe^{2+}$ chelating capacity of EPS22 was determined by measuring the iron–ferrozine complex. As shown in Table 1, at low concentrations (62.5 and 125 μg/ml), EPS22 exhibited a moderate chelating activity to $Fe^{2+}$ as compared to EDTA, a well known metal ion chelator. However, at higher concentrations, EPS22 showed strong ferrous chelating activity and chelated almost 88.5 ± 0.7%

**Table 1**
$IC_{50}$ values and antioxidant activities of EPS22 at different concentrations.

| Samples | Antioxydant activities | | | | | | $IC_{50}$ values (μg/ml) |
|---|---|---|---|---|---|---|---|
| DPPH radical-scavenging activity (%) | | | | | | | |
| Concentration (mg/ml) | 0.5 | 1 | 2 | 3 | 4 | 5 | |
| EPS22 | 54 ± 0 | 80 ± 1.41 | 80.25 ± 1.76 | 79.25 ± 2.47 | 77.9 ± 1.27 | 77.75 ± 0.35 | 450 |
| BHA | 87 | 95 | 96 | 95 | 96 | 97 | 300 |
| Reducing power activity ($OD_{700}$ nm) | | | | | | | |
| Concentration (mg/ml) | 0.5 | 1 | 2 | 3 | 4 | 5 | |
| EPS22 | 0.28 ± 0 | 0.68 ± 0.02 | 1.26 ± 0.02 | 1.26 ± 0.07 | 1.24 ± 0.02 | 1.22 ± 0.01 | 700 |
| BHA | 1.8 | 2 | 2 | 2 | 2 | 2 | 200 |
| Chelating activity (%) | | | | | | | |
| Concentration (mg/ml) | 0.0625 | 0.125 | 0.25 | 0.5 | 0.75 | 1 | |
| EPS22 | 30.5 ± 2.12 | 47.5 ± 0.7 | 77.85 ± 0.21 | 83 ± 2.82 | 88.5 ± 0.7 | 88 ± 1.41 | 170 |
| EDTA | 95 | 99 | 100 | 100 | 100 | 100 | <50 |

BHA and EDTA were used as positive controls. Values were means ± SD ($n = 2$).

of ferrous ions at 0.75 mg/ml, suggesting an effective capacity of EPS22 for iron binding.

Metal chelation is an important antioxidant property and chelating agents are effective as secondary antioxidants. In fact, they reduce the redox potential, stabilizing therefore the oxidized form of the metal ion. It was reported that the compounds with structures containing two or more of the following functional groups: OH, —COOH, C═O, —$NR_2$, —S— and —O— in a favorable structure–function configuration can show metal chelating activity [24]. According to our previous studies [8], an unusual structural feature of the EPS22 has been reported, which is the presence of three non-carbohydrate substituents, lactate, acetate and pyruvate, responsible for the anionic character of the EPS22. Therefore, besides the hydroxyl groups OH of the polysaccharide EPS22, the functional groups C═O, —COOH and —O— of the non-carbohydrate substituents, may be a possible explanation for the EPS22 high chelating activity.

In summary, the *in vitro* antioxidant capacities of EPS22, evaluated using different biochemical methods were found to be interesting.

According to previous studies, biological activities of polysaccharides can be affected by many factors including their chemical components, molecular weights and structures [24]. Qi et al. [25] reported that the acetylated ulvan derivative showed more pronounced antioxidant activities then the natural ulvan. Similar findings were reported by Yanagimoto et al. [26] where a remarkably enhancement of antioxidant activity was shown after addition of acetyl groups to pyrroles. Previously, we have demonstrated the high acetylation degree of the EPS22 under study, approximately equal to 1.5 *O*-acetyl groups per sugar [8]. Therefore, taking into account the biological importance of acetylation, this characteristic among others, may be an explanation for the high antioxidant activity of EPS22.

Moreover, the molecular weight of polysaccharides could play an important role in the antioxidant activity [22,27]. Because previous studies have shown that the antioxidant activity tends to be lower with high molecular weight polysaccharides [27], further investigation will be interesting to clarify the correlation between EPS22 molecular mass an its antioxidant activity which may be increased after its depolymerization.

Consequently, the structural features of the *P. stutzeri* exopolysaccharide EPS22, especially its monosaccharide composition and high degree of acetylation [8], may reflect its potent antioxidative power.

### 3.3. Rheological characterization of EPS22 hydrogel

To evaluate the EPS22 gel performance, its rheological behavior was studied. Measurement of viscosity as a function of shear rate (Fig. 2a) showed a shear-thinning behavior of EPS22 gel characterized by decreasing viscosity with increasing shear rate (step 1). Once the external force was removed (step 2), the EPS22 gel was able to rapidly recover and return to a higher viscosity. These results suggested good recoverability and reversibility of EPS22 gel when subjected to a shear force. Such behavior is an important feature in the use of a gelling skin care product. Rosu et al. [28] reported that products having high viscosity at low shear force might show a firm, stable and well-bodied products, with good standup properties, however, at higher shear force the viscosity decrease allowed the product, when applied, to be absorbed into the skin easily. Moreover, the ability of EPS22 gel to quickly recover and return to its initial viscosity is an important characteristic for a topical cosmetic product, to remain on the skin and not flow off after application. In addition, it must be mentioned that the zero shear viscosity of EPS22 gel, showing a material behavior at a minimum stress [28], was found to be higher than $10^3$ Pa $s^{-1}$, suggesting that EPS22 gel may be considered strong enough to have good stability during its storage.

The influences of frequency and temperature on viscoelastic characteristics were also assessed. Fig. 2b showed the EPS22 gel dynamic moduli $G'$ and $G''$ variation, as a function of oscillations frequency. It is interesting to mention that the $G'$ values were larger than $G''$ over the applied frequency, confirming the gel character and good storage stability of EPS22. Mezger [29] reported that materials having highest values of $G'$ modulus show highest stability over time.

On the other hand, the influence of temperature on defining the EPS22 gel viscoelastic characteristics is shown in Fig. 2c. EPS22 gel exhibited an elastic modulus $G'$ higher than the viscous modulus $G''$ over the whole temperature range investigated, which is an indicative of the temperature-independency of the gel. At temperature lower than about 40 °C, $G'$ and $G''$ values were almost constants. It is because a temperature interval of 20–40 °C best simulates the use of skin care products conditions [28], that the EPS22 gel characteristics being temperature independent may enhance its use in such application. Although further increase in temperature resulted in a decrease of both dynamic moduli, EPS22 gel was able to recover its initial viscoelastic properties after cooling. Interestingly, EPS22 consists in a thermoreversible and thermostable gel, once subjected to temperature variation during storage.

### 3.4. Moisture-absorption capacity of EPS22

As the EPS22 hydrogel was developed for application as a wound dressing, the evaluation of its capacity to absorb aqueous solutions is fundamental. The water absorption profile of the EPS22 hydrogel, shown in Fig. 3, showed that it exhibited a quickly absorption

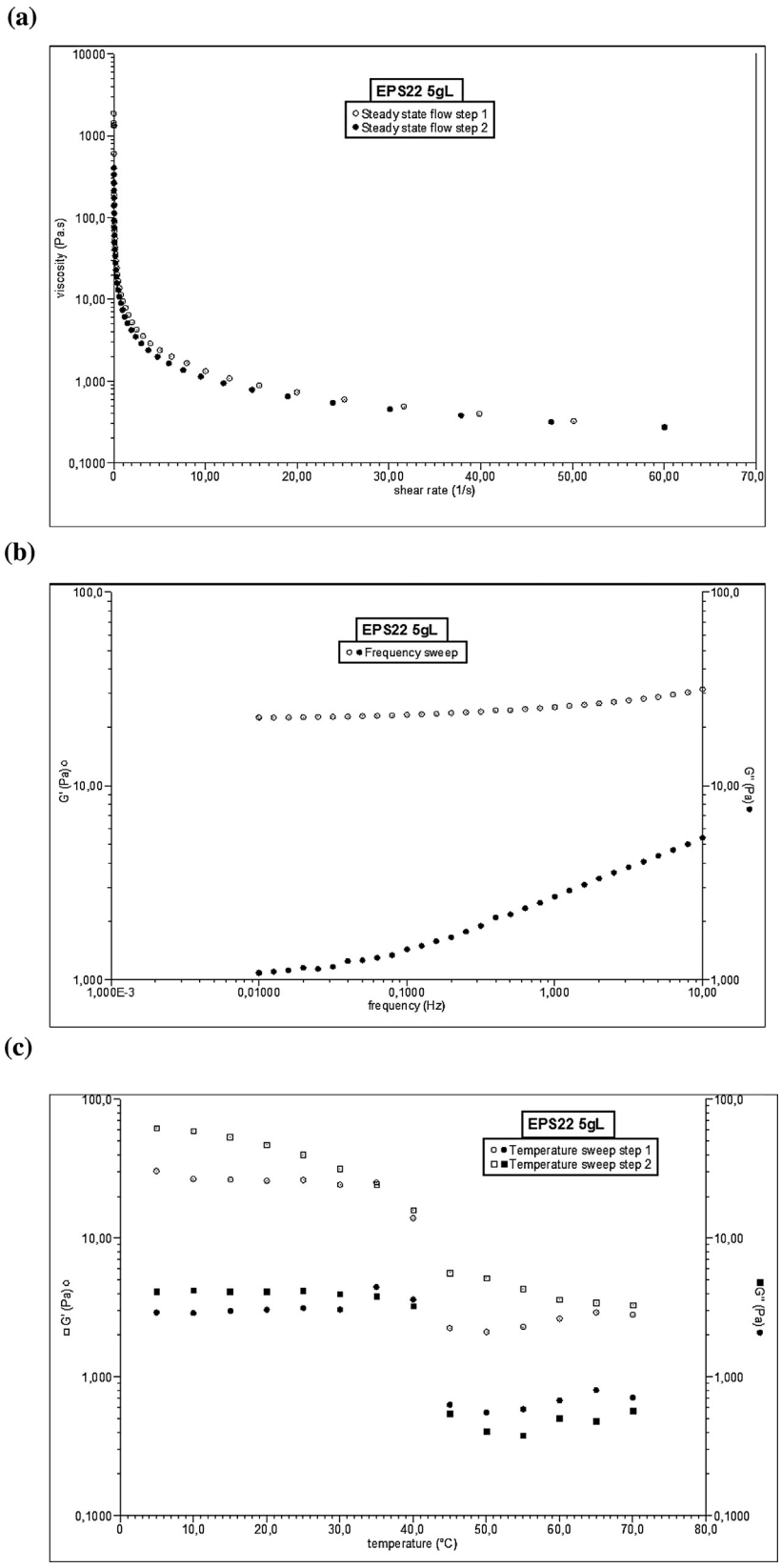


**Fig. 2.** Rheological properties of EPS22 gel (0.5% in saline solution): viscosity variation as a function of shear force (a) and dynamic moduli $G'$ and $G''$ variation as a function of frequency (b) and temperature (c).

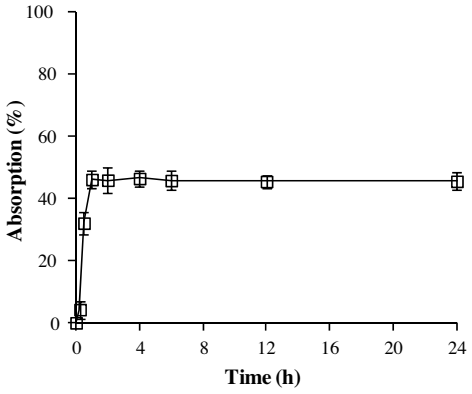


**Fig. 3.** Moisture-absorption ability of EPS22 hydrogel (0.5% in saline solution). The data presented are mean value of two experiments (mean ± SD).

of water along the first 30 min after incubation, reaching the equilibrium of 46 ± 3% in approximately 60 min.

Bolton et al. [30] reported that the use of more moisture-retentive dressings generally supports faster healing compared with less moisture-retentive dressings. Interestingly, the water uptake capacity of EPS22 hydrogel is sufficient to prevent the wound bed from accumulation of exudates.

In addition, the high absorption ability of EPS22 hydrogel may be explained by its hydrophilic nature and porous structure, which would be helpful not only for the transfer of nutrients and oxygen to the interior of tissues, but also to absorb large volume of wound exudates from the wound surface and thus to reduce infections [31].

Therefore, the strong antioxidant activity displayed by EPS22 and evaluated by the three antioxidant assays reported above, as well as its gel-forming ability with interesting mechanical properties and high moisture-absorption capacity, might be contributing factors for improved wound healing process.

### 3.5. *In vivo wound healing activity of EPS22 hydrogel*

In our wound healing rat model, a single full thickness circular skin wound (about 200 $mm^2$) was made on the back of each rat. To evaluate the wound healing ability of the EPS22 gel, we measured some parameters like the rate of wound closure, epithelialization time and hydroxyproline content in newly formed tissue along with the histological evaluation of the healed tissues.

#### 3.5.1. *General characteristic of animals*

The rat's body weights of each group were recorded before and after treatments. This parameter did not differ significantly between the studied groups (data not shown). Consequently, the different lots are homogeneous and comparable. In addition, there was minor augmentation of rat's weight after treatment in all the groups suggesting a normal growth of the rats of the study.

#### 3.5.2. *Chromatic study*

The healing process was evaluated on the basis of the wound color, the states of inflammation and cicatrization. The appearance of the wounds of the three groups was illustrated in Fig. 4a. While wounds treated with the EPS22 gel and CICAFLORA showed a bright red coloration only on the day of wound induction, this coloration is observed even after 4 days in the untreated group and hemorrhage occurred upon removal of the dressing that showed severe adhesion with the wound.

A dark red coloration was observed on the second day for both treated groups (test and standard) which gives evidence of the initiation of the healing process by the formation of blood clot with cellular debris.

On the 6th day, a healthy, clean and brown in color wound was observed in the treated groups due to the scab formation around the wound, whereas for the negative control group, pus accumulation, a sign of infection and inflammation, was observed over the damaged skin. It is well known that scabs act as a barrier to protect the wound from infections and germs. Moreover, as can be seen in Fig. 4a (day 6), the formed scab in the CICAFLORA-treated group was harder and excessive than that formed over the EPS22 gel-treated wound, leading to a more dry wound. Yang et al. [32] reported that less dry the wound was, rapidly the epidermal cells migrate, and hence the epithelialization process can be accelerated.

After 10 days, for both EPS22 gel and CICAFLORA-treated groups, most wound tissues repaired themselves with no residual scab tissue, to let appear pink blade coloration at the 12th day. However, most untreated animals still show, at the end of the experiment, an open wound with red rounding tissues.

#### 3.5.3. *Wound closure and epithelialization evaluation*

The healing process can be monitored physically by assessing the rate of the wound closure. This rate has been evaluated for each group by the determination of the unclosed wound area as a function of time, as shown in Fig. 4b.

Both the reference (CICAFLORA) and EPS22 gel-treated groups resulted in a faster reduction of the wound diameter than the negative control (physiological serum). More precisely, 50% of wound contraction was achieved on the 5th day, 6th day and the 7th day, respectively for the EPS22 gel-treated group, the reference group and the untreated group. Moreover, we noticed that in both treated groups there was approximately almost 30% reduction in the wound defect area at the first 4 days and more than 95% wound contraction was achieved on the 10th day.

In contrast, the untreated wounds healed much slowly. No wound contraction was observed at the first 4 days and only about 80% of wound contraction was achieved on day 10. After 12 days, in contrast to the treated groups in which a total closure of the wounds was achieved, the untreated animals still show an open wound (12%) at the end of the experiment. This finding is in accordance with the literature, in which it has been reported that the natural contraction of wounds takes place by the 21st post-wounding day [33].

#### 3.5.4. *Estimation of hydroxyproline content*

Skin samples, from the wound area of each rat, were also assessed for their hydroxyproline content, which gives an estimate of collagen concentration. Indeed, the relative abundance of hydroxyproline in collagen facilitates its use as a biochemical specific marker in the assessment of collagen content and thus the estimation of rates of collagen synthesis.

Generally, a rapid increase in the synthesis of this protein in the wound area was observed after injury, to restore the anatomic structure and function of normal tissues. Hence, its estimation through hydroxyproline measurement helps to understand the progress rate at which the healing process is going on in the damaged tissue.

As shown in Table 2, there was a significant increase ($P < 0.05$) in hydroxyproline level in EPS22 gel-treated group (23.36 ± 0.7 mg/g of tissue) when compared to the control group (17.91 ± 1.6 mg/g of tissue) which implies more collagen deposition and thus faster rate of wound healing process in treated groups than in the untreated one. Although the hydroxyproline content of EPS22 gel-treated group was slightly higher as compared to CICAFLORA-treated group, the level was statistically insignificant ($P < 0.1$). However,

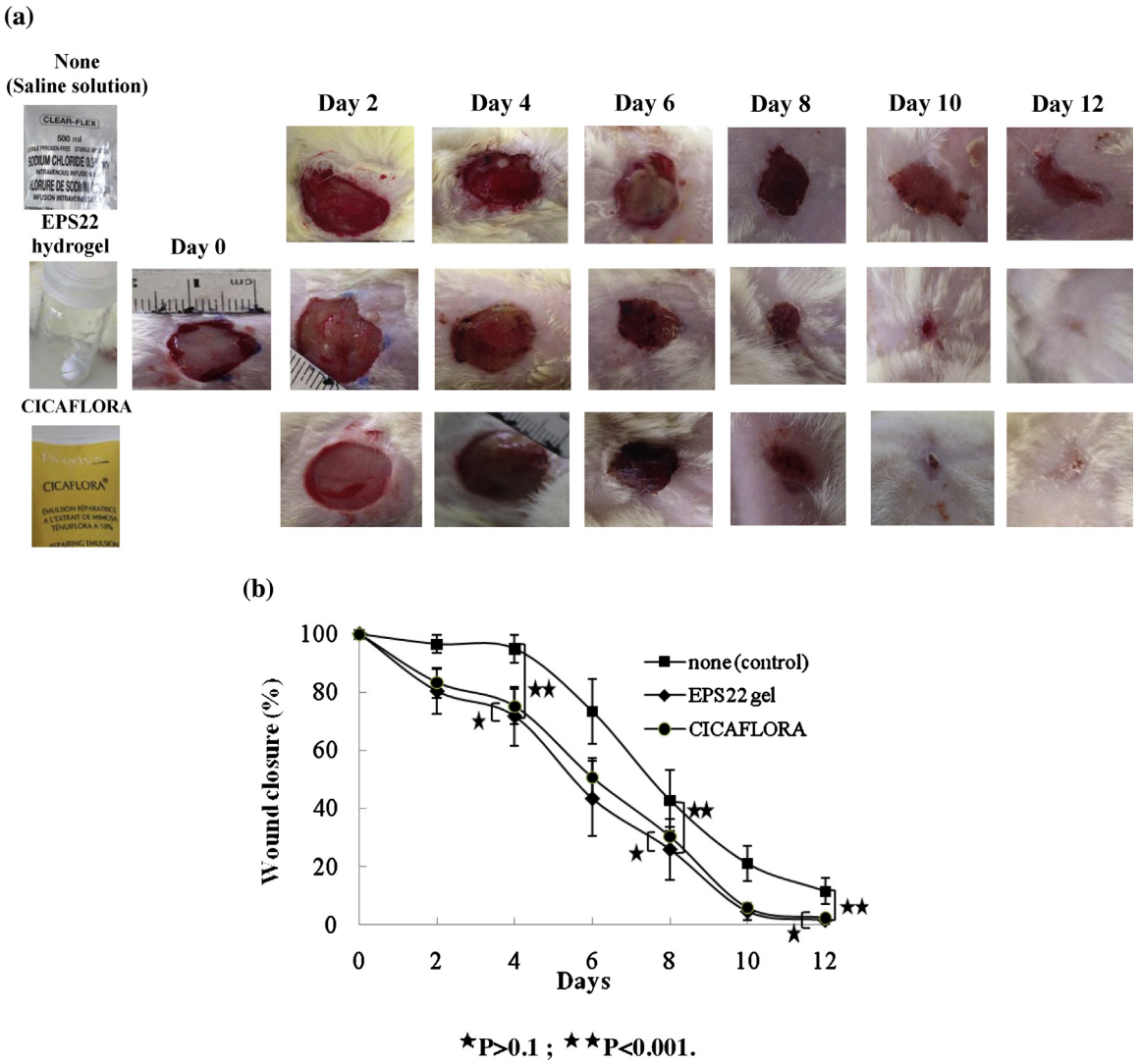


**Fig. 4.** Effects of *P. stutzeri* EPS22 gel on wound's evolution. (a) Representative photographs of excised wound, treated with saline solution, EPS22 gel and CICAFLORA. The photographs of the wound at day 0, 2, 4, 6, 8, 10 and 12 are representative of six rats in each group. (b) Reduction of wound diameter (0–12 days). Each data point represents the mean ± SD of six rats. $^{*}P > 0.1$; $^{**}P < 0.001$.

both regenerated tissues showed less hydroxyproline content than that detected in the original tissue ($P < 0.05$).

### 3.5.5. Histological evaluation

The wound tissue histology of the untreated, CICAFLORA and EPS22 gel-treated groups was studied in the 12th post-excision day.

The hematoxylin–eosin (HE) staining (Fig. 5) depicted the EPS22 and CICAFLORA-accelerated cutaneous wound healing by showing fully re-epithelialization with a well-structured layer of epidermis and faster keratinization with intraepithelial cornification (A and B). On the other hand, lack of new epidermis (E), large ulcers (U), a fibrino-inflammatory layer and moderate number of inflammatory cells were noticed in non-treated animal group confirming a much slow healing process with an inflammatory response that is still present.

In contrast to the dermis of EPS22 gel-treated groups, where vascular canals retained their normal shape and size and neovascularization was also observed, the dermis of the untreated wound revealed the presence of a pronounced hyperemia of capillary blood vessels (B and C). Moreover, we note the presence of a foreign body reaction in the untreated wounds (B and C) which might be probably the result of the gauze fibers adherence to the healing tissues, and thereafter their penetration in the dermis of the wound's tissue after being enclosed by newly grown tissue. This finding seems to be in accordance with the chromatic study, in which we have mentioned that, while gauze dressing in the EPS22 gel-treated rats showed no adherence to the wound and could be easily removed from the wound surface without causing further trauma, it was difficult to separate the gauze material from the untreated wound's tissue, thus resulting in a considerable hemorrhage and bleeding during the dressing removal.

**Table 2**
Variations in hydroxyproline content in different experimental animal groups.

| Material | Hydroxyproline (mg/g) |
|---|---|
| Original tissue | 27.2 ± 1.30** |
| Negative control | 17.91 ± 1.6** |
| CICAFLORA | 22.02 ± 0.9* |
| EPS22 gel | 23.36 ± 0.7 |

All values are mean ± SD ($n = 6$/group). Statistical analysis by ANOVA followed by Student's test.

* $P < 0.1$.

** $P < 0.05$, as compared to the EPS22 gel-treated group.

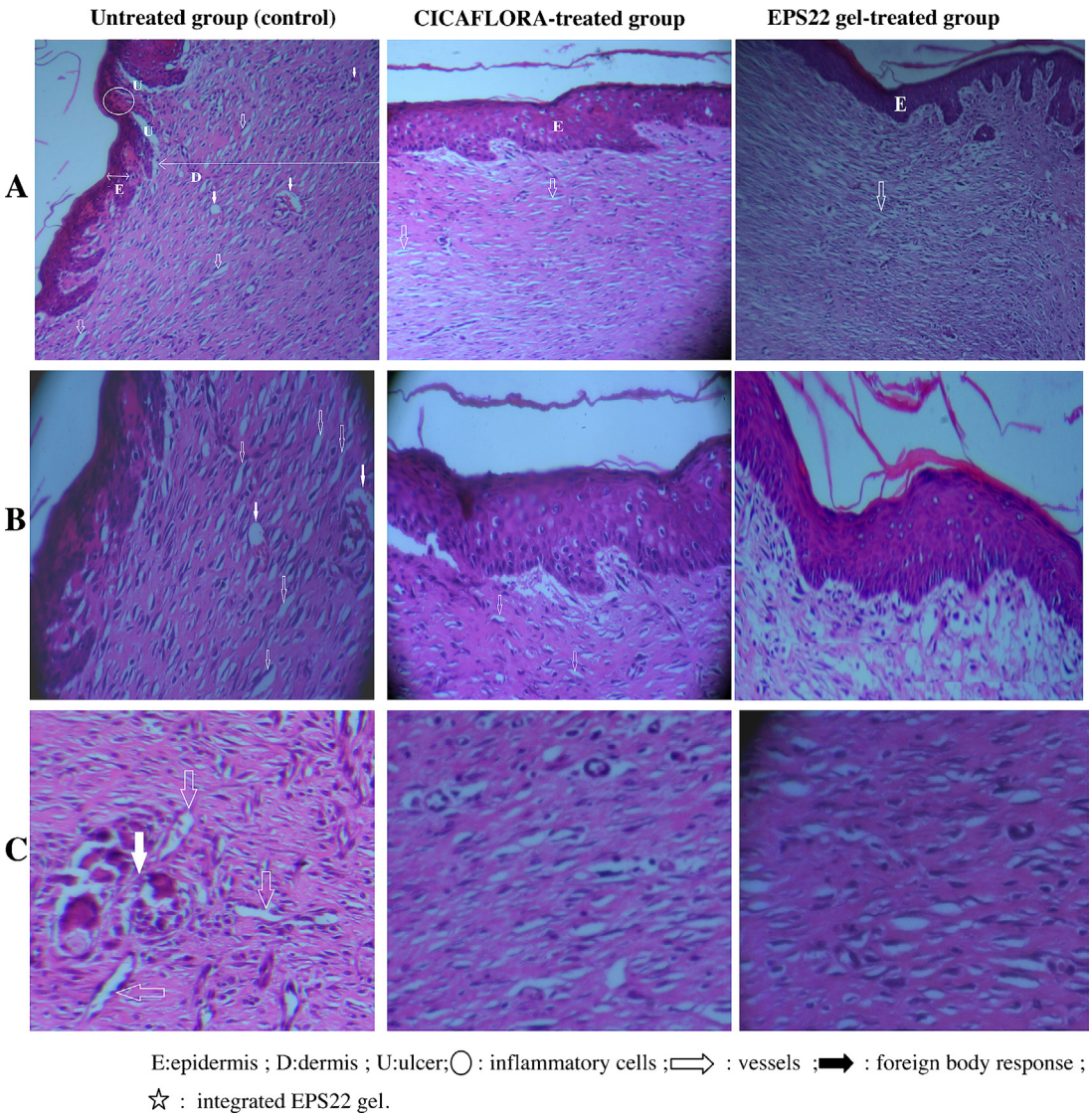


**Fig. 5.** The representative photomicrographs of the effect of EPS22 gel and CICAFLORA treatments on rat, revealing epidermal and dermal architecture of wounds on the 12th day. HE-stained histological sections are 5 mm thick and photomicrographs are taken at 100 (A), 200 (B) and 400 (C) magnification.

In the same context, the lack of an inflammatory reaction in the wound skin treated with EPS22 hydrogel with the absence of pathological abnormalities supported the histocompatibility of the EPS22 biomaterial. In addition, vacant areas were also observed inside the EPS22 gel-treated wound tissue (B), which may be related to the gel integration in the skin tissue.

The histological studies of the granulation tissue of the untreated group demonstrated a more aggregation of macrophages with moderate collagen fibers than the treated groups where a high fibroblast and collagen density with fewer macrophages were noticed (C).

Besides collagen, proteoglycans are also among the major components of connective tissue and the healing process depends on the regulated production, deposition and their subsequent maturation. Alcian blue staining (Fig. 6) revealed that the untreated wound showed dilated vessels with low blue staining intensity suggesting less deposition of proteoglycans (A) than the EPS22 gel-treated groups which exhibited a significant increase in the staining intensity, suggesting therefore the best deposition of proteoglycans. Furthermore, it is pertinent to mention here that the hydrophilic nature of proteoglycans is an essential property by which they prevent wound surface from dehydration and maintain a moist wound microenvironment. Consequently, the best proteoglycans deposition is, the more hydrated wound is, hence confirming our previous finding concerning the hydrating effect of EPS22. In addition, the high blue staining intensity observed in the biopsies of the EPS22 gel-treated wounds may be also related to the carbohydrate nature of EPS22, thus confirming the integration of the EPS22 gel in the skin tissue.

To summarize, the process of wound healing was enhanced in the animals treated with the EPS22 gel. The healing potential of the EPS22 gel was as interesting as that of CICAFLORA, a well known healer, thus indicating a clear role of this biopolymer in the enhancement of the wound healing process.

Several factors have been reported to be involved in the enhancement of the wound healing process. Süntar et al. [34] reported the beneficial and protective effects of antioxidant molecules on wound healing. Besides their role in removing products of inflammation, they counter the excess of reactive oxygen species (ROS) and proteases often formed by neutrophil accumulation in the wound area. Since ROS excess may lead to severe complications such as fibroblasts cells killing, less flexibility of skin lipids, and proteases inhibitors oxidative damage, antioxidant substances will reduce the possibility of these adverse events to

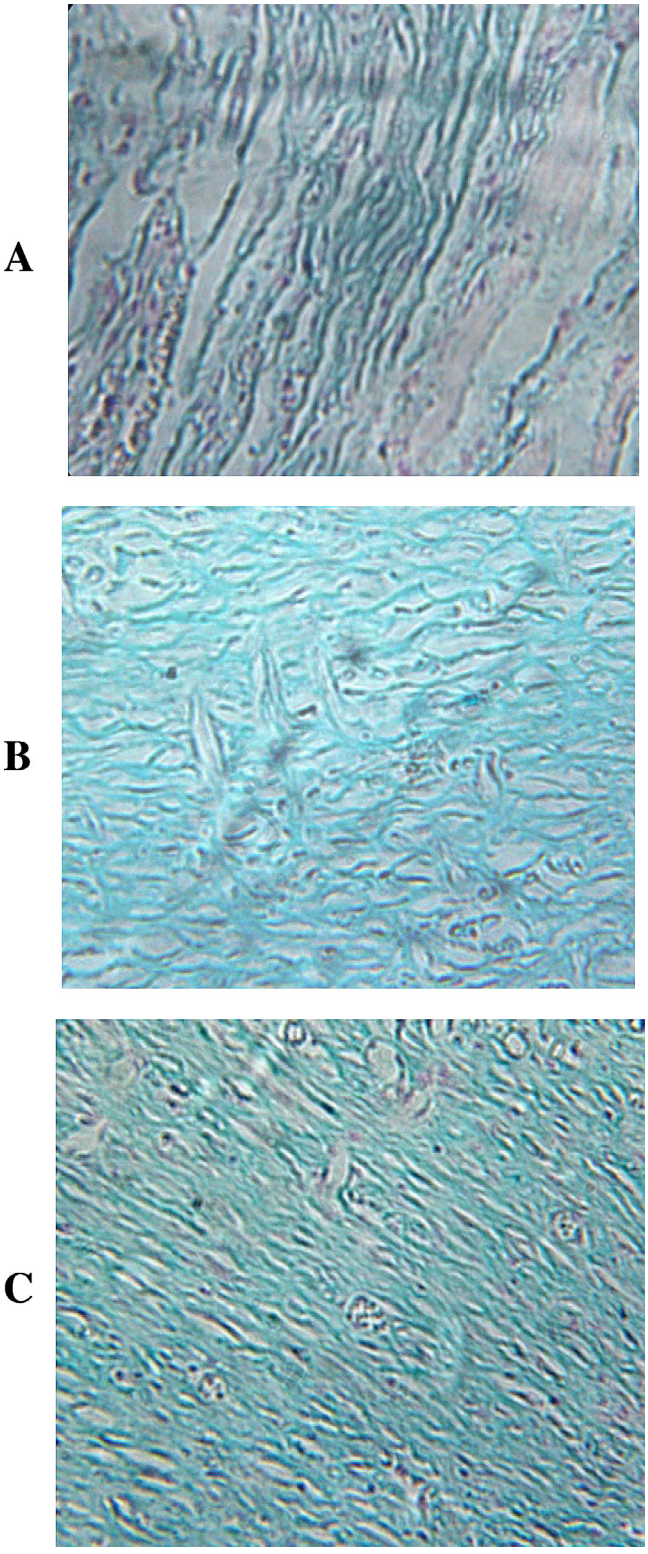


**Fig. 6.** Alcian blue stained skin sections (A–C) of tissues from the healed area of the wound of animals from different groups at 400× magnification. (A) Untreated wound showing low staining intensity of the extracellular material, indicating a low deposition of proteoglycans. (B) The skin section of rat treated with CICAFLORA cream showing higher amount of extracellular glycoside material when compared to the untreated one. (C) Wound tissue section of rat treated with the EPS22 gel. The staining intensity of Alcian blue was the highest indicating the best deposition of proteoglycans.

occur. Therefore, the antioxidant property of EPS22 may be among the most efficient contributing factors for improved wound healing.

Besides, the beneficial effect of EPS22 in promoting wound healing may be also attributed to its gelling ability as well as the mechanical strength of the corresponding gel. Kean and Thanou [35] reported that the high water content of hydrogels makes them compatible with the majority of living tissues, besides their capacity to maintain a moist environment. Moreover, as reported by Drury and Mooney [36], the microstructure of hydrogel can provide a 3D structure for cell adhesion, proliferation, transportation of cytokines, nutrients and metabolic wastes. Moreover, the ability of EPS22 hydrogel to absorb moisture is also an important factor for wound healing that determines its ability to absorb exudates from the wound, avoiding maceration and maintaining a moist environment.

## 4. Conclusion

The present work explored the potential use of an exopolysaccharide EPS22 produced by *P. stutzeri* AS22 as a hydrogel wound dressing. EPS22 was found to exhibit strong antioxidant activities evaluated by three tests. Interestingly, EPS22 gel fulfills the rheological and moisture-absorption properties, required for a product to be suited for pharmaceutical and cosmetic use.

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## References

[1] F. Croisier, C. Jérôme, Eur. Polym. J. 49 (2013) 780.
[2] G. Gainza, J.J. Aguirre, J.L. Pedraz, R.M. Hernández, M. Igartua, Eur. J. Pharm. Sci. 50 (2013) 243.
[3] F. Donot, A. Fontana, J.C. Baccou, S. Schorr-Galindo, Carbohydr. Polym. 87 (2012) 951.
[4] S. Ye, F. Liu, J. Wang, H. Wang, M. Zhang, Carbohydr. Polym. 87 (2012) 764.
[5] M. Yasuhiro, K. Yoshimitsu, J. Biomater. Sci. Polym. Ed. 21 (2010) 715.
[6] S. Guo, L.A. Dipietro, J. Dent. Res. 89 (2010) 219.
[7] G. Mohammad, V.K. Mishra, H.P. Pandey, Digest, J. Nanomat. Biostruc. 3 (2008) 159.
[8] H. Maalej, C. Boisset, N. Hmidet, L. Buon, A. Heyraud, M. Nasri, Carbohydr. Polym. 112 (2014) 404.
[9] P. Bersuder, M. Hole, G. Smith, J. Am, Oil Chem. Soc. 75 (1998) 181.
[10] A. Yildirim, A. Mavi, A.A. Kara, J. Agric. Food Chem. 49 (2001) 4083.
[11] P. Carter, Anal. Biochem. 40 (1971) 450.
[12] B. Balakrishnan, M. Mohanty, P.R. Umashankar, A. Jayakrishnan, Biomaterials 26 (2005) 6335.
[13] I. Teot, S. Meaume, O. Dereure, Plaies et cicatrisations au quotidien, Sauramps médical, Montpellier, 2001.
[14] A. Fikru, E. Makonnen, T. Eguale, A. Debella, G. Mekonnen, J. Ethnopharmacol. 143 (2012) 469.
[15] K.H. Lee, T.G. Tong, J. Pharm. Sci. 57 (1968) 1042.
[16] J.F.A. McManus, R.W. Mowry, Staining methods, Histologic and Histochemical, Harper Raw, New York/Evanston/London, 1965.
[17] J.E. Scott, J. Dorling, Histochemistry 5 (1965) 221.
[18] M.W. Tibbitt, K.S. Anseth, Biotechnol. Bioeng. 103 (2009) 655.
[19] N. Annabi, J.W. Nichol, X. Zhong, C. Ji, S. Koshy, A. Khademhosseini, F. Dehghani, Tissue Eng. B: Rev. 16 (2010) 371.
[20] T.C.T. Lo, C.A. Chang, K.H. Chiu, P.K. Tsay, J.F. Jen, Carbohydr. Polym. 86 (2011) 320.
[21] Y. Chen, M.Y. Xie, S.P. Nie, C. Li, Y.X. Wang, Food Chem. 107 (2008) 231.
[22] W. Raza, W. Yang, Y. Jun, F. Shakoor, Q. Huang, Q. Shen, Carbohydr. Polym. 90 (2012) 921.
[23] W. Raza, K. Makeen, Y. Wang, Y.C. Xu, Q.R. Shen, Bioresour. Technol. 102 (2011) 6095.
[24] H. Qi, T. Zhao, Q. Zhang, Z. Li, R. Xing, J. Appl. Phycol. 17 (2005) 527.
[25] H. Qi, Q. Zhang, T. Zhao, R. Hu, K. Zhang, Z. Li, Bioorg. Med. Chem. Lett. 16 (2006) 2441.
[26] K. Yanagimoto, K.G. Lee, H. Ochi, T. Shibamoto, J. Agric. Food Chem. 50 (2002) 5480.

[27] T.T. Zhao, Q.B. Zhang, H.M. Qi, H. Zhang, X.Z. Niu, Z.H. Xu, Z.E. Li, Int. J. Biol. Macromol. 38 (2006) 45.
[28] A. Rosu, S. Bistriceanu, C. Ibānescu, O.M. Daraba, M. Lungu, Cellul. Chem. Technol. 47 (2013) 359.
[29] T.G. Mezger, in: T.G. Mezger (Ed.), The Rheology Handbook for Users of Rotational and Oscillatory Rheometers, Coatings Compedia, 2006, pp. 80–88.
[30] L.L. Bolton, K. Monte, L.A. Pirone, Ostomy Wound Manag. 46 (2000) 51S.
[31] A.H. Liu, F.T. Jiang, Mater. Sci. Eng 25 (2007) 826.
[32] X. Yang, K. Yang, S. Wu, X. Chen, F. Yu, J. Li, M. Ma, Z. Zhu, Radiat. Phys. Chem. 79 (2010) 606.
[33] G. Winter, Nature 200 (1963) 378.
[34] I. Sv̈ntar, E.K. Akkol, L. Nahar, S.D. Sarker, Free. Radic. Antioxid. 2 (2012) 1.
[35] T. Kean, M. Thanou, in: P.A. Williams (Ed.), Renewable Resources for Functional Polymers and Biomaterials, Polysaccharides, Proteins and Polyesters, Royal Society of Chemistry, London, 2011, p. 292.
[36] J.L. Drury, D.J. Mooney, Biomaterials 24 (2003) 4337.